Nephrin expression, localization and role as an interacting molecule

Emphasis on kidney glomerular podocytes

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Natura nihil facit frustra,
non deficit in necessariis,
nec abundat in superfluis.

- Aristoteles (384-322)

Contents

List of original publications

I. Ahola H, Wang SX, Luimula P, Solin ML, Holzman LB, Holthofer H: Cloning and expression of the rat nephrin homolog**.** *Am J Pathol* 1999 155 (3): 907-13

II. Holthofer H, Ahola H, Solin ML, Wang S, Palmen T, Luimula P, Miettinen A, Kerjaschki D: Nephrin localizes at the podocyte filtration slit area and is characteristically spliced in the human kidney. *Am J Pathol* 1999 155 (5): 1681-7

III. Luimula P**,** Ahola H, Wang SX, Solin ML, Aaltonen P, Tikkanen I, Kerjaschki D, Holthofer H: Nephrin in experimental glomerular disease. *Kidney Int* 2000 58 (4): 1461-8

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V. Ahola H, Heikkilä E, Åström E, Inagaki M,Izawa I, Pavenstädt H, Kerjaschki D and Holthofer H: A Novel Protein, Densin, Expressed by Glomerular Podocytes. J Am Soc Nephrol 2003 14 (7): 1731-7

* These two authors have contributed equally to the study

Abbreviations

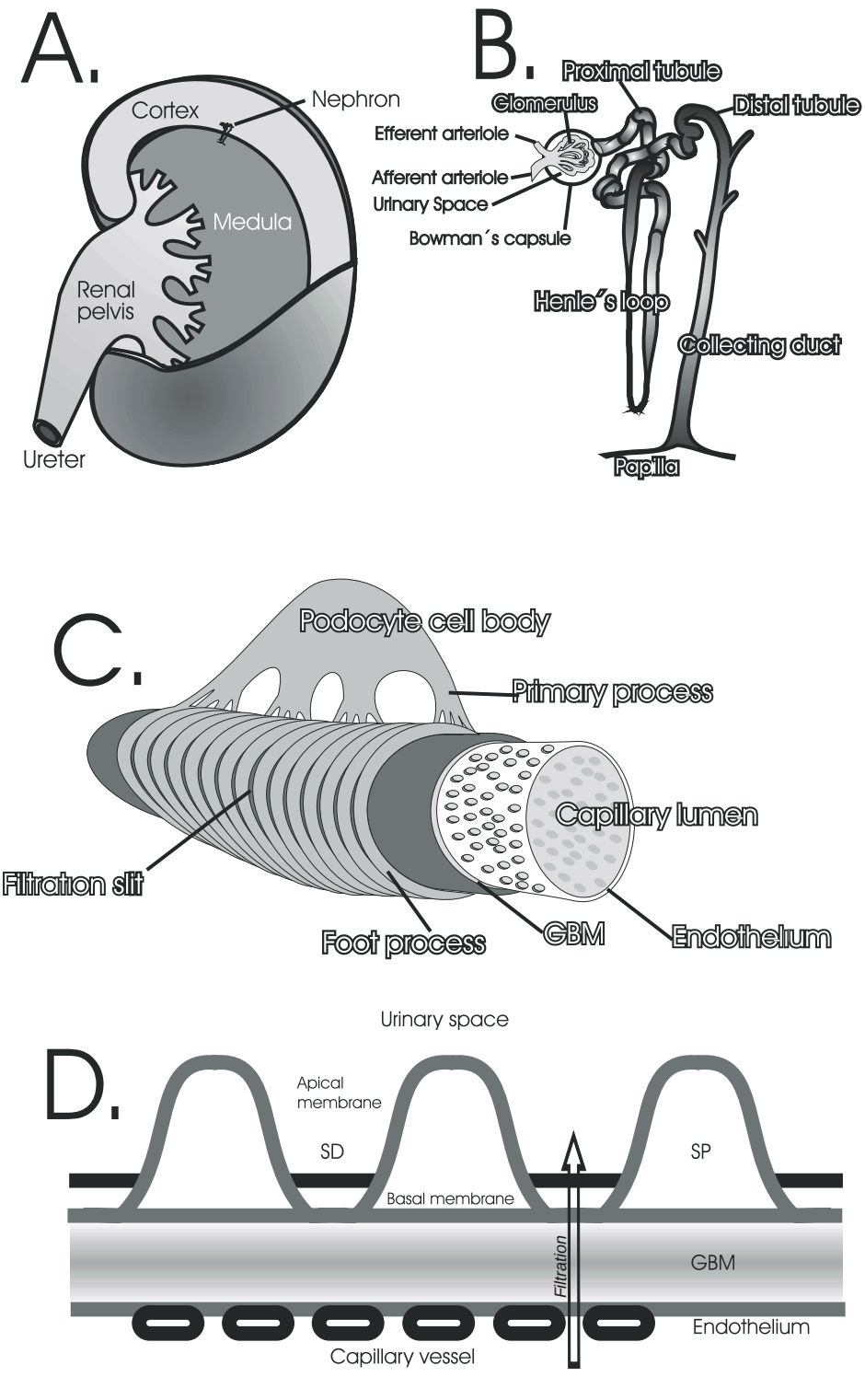
ACTN4	alpha-actinin-4
ARF	acute renal failure
ATP	adenosine triphosphate
BB	brush border
GBM	glomerular basement membrane
C3	complement component 3
C5b-9/MAC	membrane attack complex
CaMKII	Ca^{2+}/calmodulin -dependent protein kinase II
CD2AP	CD2 associated protein
cDNA	complementary DNA
CNF	congenital nephrotic syndrome of the Finnish type
DNA	deoxyribonucleic acid
EM	electron microscopy
ER	endoplasmic reticulum
ERM	ezrin-radixin-moesin protein family
FITC	fluorescein isothiocyanate
Ig	immunoglobulin
LAP	leucine-rich repeats and PDZ domains
L-NAME	L-N^G-nitroarginine-methylester
LRR	leucine-rich repeats
Mab 5-1-6	monoclonal antibody 5-1-6
MAGUK	membrane-associated guanylate kinase
MAGUIN-1	MAKUG interacting protein 1
MCN	minimal change nephropathy
MGN	membranous glomerulonephritis
mRNA	messenger RNA
NEPH	nephrin-like protein
NHE3	Na^+/H^+ -exhanger 3
NHERF2	Na^+/H^+ -exhanger regulatory factor 2
NPRAP	delta-catenin/neural plakophilin –related armadillo repeat protein
MW	molecular weight
NPHS1	nephrin gene
NPHS2	podocin gene
NS	nephrotic syndrome
PA	puromycin aminonucleoside
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDZ	PSD-95/Dlg-A/ZO-1 domain
PSD	postsynaptic density
PSD-95	PSD protein 95
RGD	arginine-glysine-asparagine tripeptide
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SD	slit diaphragm
TRITC	tetramethylrhodamine isothiocyanate
WT1	Wilm’s tumour 1
ZO-1	zonula occludens-1

Introduction

Proteinuria, the loss of proteins in urine, is a key marker of dysfunction in kidney glomerulus. The degree of proteinuria in patients with renal disease correlates closely with the severity of damage to the glomerulus. However, despite the pathophysiological relevance of this marker, the exact mechanisms of glomerular damage resulting in overt proteinuria remain poorly understood.

Glomerular filtration barrier is composed of three separate layers: endothelium, glomerular basement membrane (GBM) and glomerular epithelial cells, podocytes. Along with the recent molecular findings, podocytes are emerging as a key cell type involved in glomerular filtration barrier. The ability of podocytes to form foot processes with unique intercellular slit diaphragms (SD) reflects a special organization involved in glomerular filtration. The protein complex forming the SD has been basically unknown until the cloning of human *NPHS1*, the gene encoding nephrin protein. Mutations in *NPHS1* cause congenital nephrotic syndrome of the Finnish type (CNF), manifesting e.g. as massive proteinuria and the lack of SD.

The purpose of this thesis was to shed light on the role of SD in glomerular filtration by studying the function of nephrin. The precise localization of nephrin in the kidney and its expression profile in other tissues were investigated. Also, novel nephrin-associated proteins were searched. Nephrin involvement in renal diseases was studied by employing two experimental rat models of proteinuria: puromycin aminonucleoside (PA) nephrosis and Heyman nephritis (HN).


A.
Cortex
Nephron
Medula
Renal pelvis
Ureter
B.
Proximal tubule
Glomerulus
Distal tubule
Efferent arteriole
Afferent arteriole
Urinary Space
Bowman´s capsule
Henle´s loop
Collecting duct
Papilla
C.
Podocyte cell body
Primary process
Capillary lumen
Filtration slit
Foot process
GBM
Endothelium
Urinary space
D.
Apical membrane
SD
SP
Basal membrane
Filtration
GBM
Endothelium
Capillary vessel

Review of the literature

1.Glomerular filtration

The kidneys are a pair of organs responsible for the filtration and excretion of metabolic waste products as well as for the maintaining water and pH balance of the body. The filtration (urine formation) regulates body fluid balance by affecting the electrolyte contents, osmolarity as well as the acidity of body fluids, and takes place in the kidney's functional units, nephrons (**Figure 1A**). Each of the approximately one million nephrons in the human kidney consists of the renal corpuscle with the Bowman's capsule and the glomerulus, a proximal tubulus, the Henle's loop, a distal tubulus, and a collecting duct (**Figure 1B**) (1).

Glomerular filtration takes place in the outer zone, i.e. cortex, of the kidney (**Figure 1A**). The afferent artery, which imports 1.1 litres of blood every minute (approximately 20 % of the total cardiac output, which is more than in the brain) to the glomerulus, further divides into 5 – 7 smaller capillary branches forming the tuft-like structure of the glomerulus surrounded by the urinary space and the Bowman's capsule (**Figure 1B**). Thus, the glomerulus maintains filtration by forming a barrier between blood circulation and the tubular system, which is responsible for the formation of the final urine. Even though approximately 180 l of ultra filtrate, i.e. primary urine, is produced every day, the final urine volume is only 1-1.5 l/day. This is due to the extensive reduction of approximately 99 % of primary urine during the reabsorbtion in the proximal and distal tubuli located downstream from the glomerular filtration barrier(1).

1.1 Endothelial layer

The first step of filtration takes place through the endothelial cell layer of the capillary lumen wall (**Figure 1C and D**). There are numerous small openings, fenestrations, in the endothelium, which are filled with negatively charged glycoproteins and lipids (1). Because the pore diameter is approximately 75 nm, blood cells stay in circulation, whereas water and many macromolecules, e.g. albumin, flow without restraint through the pores (2).

Figure 1. Description of the glomerular filtration machinery in the kidney. A) Dissected kidney shows two separated layers: the outer cortex and the inner medulla B) A nephron is responsible for urine formation; glomerulus maintains the filtration by forming a filtration barrier between the urinary space and blood circulation. C) Glomerular capillary lumen is covered by fenestrated endothelium, glomerular basement membrane (GBM) and podocyte-derived foot (secondary) processes. D) Podocyte foot processes are interconnected across the filtration slit by a special structure, a slit diaphragm (SD). Molecular composition of the SD has been presented in detail in Figure 5.

1.2 Basement membrane

The second layer in the filtration barrier is the negatively charged glomerular basement membrane (GBM) (**Figure 1C and D**). It presumably prevents the flow of charged molecules into the primary urine (3). In addition to charge selectivity, GBM forms a size-selective barrier for particles with a size similar or larger to that of albumin (MW 68 kDa). GBM is composed mainly of Type IV collagen and laminin, but contains also heparan sulphate proteoglycans, fibronectin and entactin/nidogen (for reviews, see (4, 5))

1.3 Epithelial layer

The last filter is composed of the visceral epithelial cells called podocytes. Podocytes have a highly specialized structure with major (primary) processes and foot (secondary) processes that extend from the cell body, which has been postulated to lie freely in the urinary space (**Figure 1C and D**). A prominent nucleus of the podocyte is located in the cell body that has also been shown to be abundant in mitochondria (1). Contractile secondary processes, the tentacle-like ends of the primary processes, cover the basement membrane on the urinary side of the capillaries (**Figure 1C**). In contrast to the cell body, the foot processes contain only few organelles. The foot processes stabilize the shape of the glomeruli (6). They also maintain the filtration surface of the podocytes by a special structure, the slit diaphragm (SD), which interconnects the adjacent foot processes. In addition to producing the structural components of the SD, podocytes together with the cells of the mesangium have been proposed to synthesize the majority of the GBM components. In many pathological conditions associated with proteinuria, a continuous cytoplasmic band along the glomerular basement membrane replaces the foot processes. This phenomenon is commonly referred to as foot process fusion or effacement (1). Importantly, due to the high degree of differentiation of mature podocytes, like neurons, they have not been shown to proliferate (7).

1.4 Mesangial tissue

The mesangial tissue between the glomerular capillaries consists of mesangial cells and an intercellular matrix. Mesangial cells contain contractile primary processes that attach directly to the capillaries and regulate the diameter of the capillary as well as provide structural support for the glomeruli by contractions. The cell contractions are stimulated by many vasoactive agents and the relaxations caused by e.g. dopamine and atrial peptides (8). Both processes affect directly the blood pressure. Mesangial cells are abundant in myosin and actin filaments and have functional properties, such as phagocytic activity. They also produce for instance matrix components, hormones, cytokines, enzymes, and lipids (9, 10).

2. Filtration barrier dysfunction and proteinuria

Normally small and neutral proteins pass the filtration barrier freely, this not being a problem since active reabsorbtion in the downstream tubular system returns the majority of the proteins back into circulation. Under proteinuric conditions proteins that are normally kept in the blood circulation leak into the urine (11). This results in an increased amount of total protein load in the tubular system, which in turn overloads the tubular reabsorbtion capacity and proteins pass freely into the collecting duct. Autoimmune diseases, local inflammations, toxins, metabolic products and genetic mutations may cause these conditions. Protein leakage into the urine is often due to dysfunction of the selective glomerular filtration barrier with functional or structural changes in the corresponding proteins. However, also inhibition of the tubular reabsorbtion by for instance the presence of metal ions, leads to proteinuria (12).

As a result of proteinuria, the protein concentration in plasma is reduced. In clinical work the levels of one of the most abundant plasma proteins, albumin, can be measured in order to find out the severity of proteinuria. Limited amounts of albumin may normally be detected in the urine whereas increasing concentration points at increasing proteinuria. Albumin is one of the most important components maintaining osmotic balance between the cells and interstitium. Thus, in severe or extended albumin loss/proteinuria this balance is disturbed, leading to oedema (12).

3.Animal models as the prototypes of human glomerular diseases

Nephrotic syndrome (NS) is reflected by increased amounts of proteins in the urine. Other clinical features of NS are hypoalbuminemia, hypercholesterolemia and oedema. Primary or idiopathic NS may be caused e.g. by minimal change nephropathy, focal segmental glomerulosclerosis, mesangial proliferative glomerulonephritis, IgA nephropathy, membranous glomerulonephritis and membranoproliferative glomerulonephritis (13, 14). Secondary NS can be consequence of i.e. complications of other diseases, toxic or infectious agents. One important group, even though rare, of NS is the congenital diseases including e.g. Denys-Drash syndrome (15), congenital nephrotic syndrome of the Finnish type (see chapter 4.1) and disorders of the GBM (16).

Experimental animal models are widely used to study the molecular mechanisms of a broad variety of diseases *in vivo*. During recent years some of the most commonly used models are knock-out and transgenic mice with a proven capacity to reveal the functional properties of the molecules in the tissue. In addition to mouse models, experimental rat models are widely used and are suitable to obtaining functionally relevant data, especially in glomerular injury

research. The advantage of the rat models is that they have been carefully characterized during the past decades and allow collection of time-dependent tissue samples in the due process leading to disease.

3.1 Minimal change nephropathy and puromycin aminonucleoside nephrosis

3.1.1 Minimal-change nephropathy

Minimal-change nephropathy (MCN) belongs to the heterogenous group of disorders called primary glomerular diseases, in which the glomeruli are the only or the principal target. MCN is the most common cause of primary nephrotic syndromes (NS) in children between 2 and 6 years of age. Typical findings are heavy proteinuria, hypoalbuminemia and hyperlipidemia. Microscopic haematuria is observed in about 20 percent of cases. Serious haematuria and hypertension are relatively uncommon (14). Only minor, if any, glomerular changes are visible under light microscopy, but in electron microscopy (EM) flattening of foot processes can be observed (17). Charge alterations in the filtration barrier of the MCN glomeruli have been detected (18); other findings include the presence of lipid droplets in proximal tubules. The pathogenesis of MCN is still unclear, but it has been suggested that MCN might be an autoimmune disease (14).

3.1.2 Puromycin aminonucleoside nephrosis

Puromycin aminonucleoside (PA) nephrosis is a widely used animal model for proteinuria. In the PA nephrosis model, the morphological and functional changes are similar to human minimal change nephropathy (MCN). After a single intravenous injection of PA into rat, proteinuria starts at day 3-4 and is at its maximum at day 10. The kidney damage is reversible, and normal kidney function is regained at around day 25 (19). Morphological changes include dilatations of tubuli, thickening of the GBM, altered or lost SD and later on, detachment of the epithelium from the GBM. Fusion and retraction as well as cytoskeletal rearrangement of the foot processes of podocytes are typical findings in PA nephrosis (20). Furthermore, a change in the expression and localization of several podocyte proteins has been observed (see Chapter 5).

3.2 Membranous glomerulonephritis and Heymann nephritis

3.2.1 Membranous glomerulonephritis

Membranous glomerulonephritis (MGN) is included in the primary glomerular diseases. The majority of MGN patients are adults of over 30 years of age. Severe proteinuria and microscopic haematuria are typical hallmarks in MGN. In light microscopy analysis, the capillary wall is found thickened, and it trespasses into the capillary lumen. In EM studies, this has been shown to be caused by subepithelial and intramembranous immune complex deposits (14). The deposits alter the organization of the podocyte foot processes by distorting the structure. Intracapillary thrombosis is also commonly detected in MGN but it is thought to be a consequence of the MGN itself. Immunoglobu-

lin G and complement component C3 are frequently found in the capillary loops of MGN glomeruli. Even though the exact pathogenesis of MGN is unclear, some suggestions of how the immune complex deposits develop have been made (21). The deposits may be circulating immune complexes accumulating in the glomeruli or they may arise due to an *in situ* reaction between circulating antibodies and antigens in the glomerulus (14).

3.2.2 Heymann nephritis

Heymann nephritis (HN) of the rat is a model of immune complex-mediated glomerular damage (22). The HN model is analogous with the human MGN. HN is induced by injecting rats with proximal tubular brush border (BB) antigen, Fx1A, (active HN) or the anti-Fx1A antibody (passive HN). Accumulation of antigen-antibody immune complexes on the podocyte surface activates complement C5b-9 (also called membrane attack complex, MAC) causing proteinuria (23, 24). Simultaneously with the deposit-formation of cell-damaging agents, reactive oxygen species (ROS), hydroperoxides, are produced. Thus, the proteinuria in HN could be reduced by inhibition of lipid peroxidation by treatment with the antioxidant agent probucol (23, 25).

The protein affected at inducing HN is gp330, also called megalin, a glygoprotein of the lipoprotein receptor family. It is expressed in clathrin-coated pits at the bases of foot processes of podocytes and proximal tubular cells, and it has been shown to function as an anchored receptor in the endocytotic pathway (26).

3.3 Mercuric chloride model

The kidneys are the primary organs where inorganic mercuric chloride ($HgCl_2$) is taken up. Thus, acute renal failure (ARF) in rats can be induced by exposure to $HgCl_2$. Mercuric ions bind with high affinity to sulfhydryl–containing molecules and form conjugates with e.g. glutathione and proteine cysteines in the kidneys. Accumulation of mercuric ions is mainly seen in the proximal tubule, but also other parts of the nephron participate in the absorbtion and gathering of mercuric ions (27). The primary effect of $HgCl_2$ is the induction of oxidative processes (28). It is promoted by reduced production of counter-balancing molecules (protecting agents), e.g glutathione, and increased levels of the free oxygen radical, hydrogen peroxide. Free oxygen radicals can induce oxidation of lipids. Mercuric chloride reduces adenosine triphosphate (ATP) synthesis in mitochondria and is shown to induce apoptosis by causing mitochondrial dysfunction (29).

4. NPHS1 gene and its protein product nephrin

4.1 NPHS1 is mutated in congenital nephrotic syndrome of the Finnish type (CNF)

Mutations in the *NPHS1* gene lead to congenital nephrotic syndrome of the Finnish type (CNF) (30). CNF is an autosomal recessive renal dis-

ease (31-33) and is mainly found in Finland with an approximate incidence of 1/10000 of live births (34). Over 90% of *NPHS1* mutations in the Finnish population are either Fin_{major} with an early stop codon in exon 2 or Fin_{minor} with a stop codon in exon 26. A variety of different mutations leading to various clinical manifestations has been described also in other countries (35-37). In earlier times, CNF led to death during the first months of life, but in 1984, a new treatment protocol was introduced. It consists of early nephrectomy and dialysis until the body weight rises to an acceptable level for transplantation (38). This treatment removes all symptoms apparently with no later manifestations in other organs (39). The clinical features of CNF are massive proteinuria already *in utero*, oedema, enlarged placenta and low birth weight (31, 40, 41). Detection of high levels of alpha-fetoprotein in the amniotic fluid and in the maternal circulation has been used for prenatal diagnosis of CNF (42-44). However, after the finding of the causative *NPHS1* gene defect, the diagnosis is not infallible without mutation analysis (45). Interestingly, after transplantation, 20% of CNF patients manifested with a recurrence of the nephrotic syndrome, which has been shown to occur due the presence of nephrin-specific circulating auto-antibodies (46, 47).

CNF kidneys are twice as heavy compared to normal, and the number and size of glomeruli are significantly increased (48-50). At the same time, the number of podocytes is decreased and the podocytes show effacement of the foot processes and absence of slit diaphragms (43). The glomeruli are also more dense in CNF kidneys than in normal kidneys (50-52). Other histological findings include dilated proximal tubuli, mesangial hypercellularity with expansion of the mesangial area, inflammation, thickened basement membrane and thickened parietal epithelial cell layer of Bowman's capsule (33, 43, 44, 50, 51, 53).

NPHS1 gene locates to chromosome locus 19q13.1 (54, 55). The gene spans about 26 kb of DNA sequence and contains 29 exons, ranging from 25 bp to 216 bp in length (35), and a recently found additional exon 271 bp in length located 3.7 kb upstream of the ATG translation initiation codon in exon 1. Thus, the originally reported exon 1 has been named exon 1A, and the additional exon, exon 1B (56). The gene produces a 4,3-kb long mRNA, which is translated to a protein called nephrin.

4.2 Nephrin protein

4.2.1 Nephrin is a member of the immunoglobulin superfamily

The predicted domain structure of nephrin classifies it as a member of the immunoglobulin (Ig) superfamily (**Figure 2**). Moreover, a new subfamily of nephrin-like (NEPH) molecules including (in addition to nephrin) NEPH1, NEPH2 and NEPH3/filtrin has recently been

described (57, 58). Structural counterparts of these proteins have also been identified in *Drosophila* (59).

The group of molecules in the Ig superfamily shares a common structural feature, the Ig homology domain, coded by exons from 2 to 20 of the nephrin gene. Ig–like domains are suggested to be involved in protein-protein and protein-ligand interactions. The members of this family are found at different levels of phylogeny, from insects to man. The Ig-superfamily has been shown to consist of hundreds of proteins with different functions, many of them serving as cell surface receptors involved in immune reactions and binding of viruses and growth factors. Receptors of the Ig-superfamily function abundantly in conjunction with other cell adhesion proteins including members of the integrin family, cadherins and selectins. The finding of involvement of Ig-superfamily proteins in signal transduction is also supported by their ability to associate with cytoplasmic non-receptor kinases (60). While the precise functions of nephrin remain to be characterized, it is probable that it shares the general functions like adhesion molecule and receptor function with the other members of the Ig family. As an example, the Ig-superfamily includes proteins like antibodies, leukocyte function antigen-2 (LFA-2 or CD-2) (61) and -3 (LFA-3 or CD58) (62), intercellular adhesion molecules (ICAMs) (63-65), vascular cell adhesion molecule-1 (VCAM-1) (66) and platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31) (67). The members of this family have also been found to function in T-cell antigen and/or MHC recognition (68-70).

4.2.2 Domain composition

4.2.2.1 Signal sequence

The first 22 amino acids encoded by exon 1A of the human nephrin gene form the amino-terminal signal sequence, which is cleaved by a peptidase in the lumen of the endoplasmic reticulum (ER) (30). However, the recently reported exon 1B does not have this signal peptide resulting in an altered expression profile of nephrin (56).

4.2.2.2 Immunoglobulin-like domains

The eight Ig-like domains of the extracellular part of nephrin are of C2 type. The Ig C2 domains are from 80 to 100 amino acids in length and have a folding pattern distinct from the other Ig superfamily domains C1 and V (71). The C2-type subfamily includes glycoproteins and other unrelated proteins with Ig domains, e.g. carcinoembryonic antigens (72) and fibroblast growth factor receptors (73).

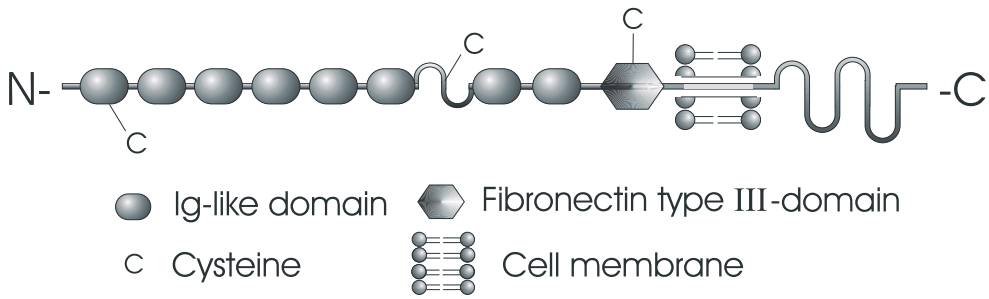


Figure 2.Domain composition of nephrin. Nephrin sequence consists of an N-terminal extracellular part, a transmembrane domain and a C-terminal intracellular domain.

4.2.2.3 Free cysteine residues
Thiol group-containing cysteines can form disulphide bonds with each other thus cross-linking and stabilizing the folded protein chain. In addition to the cysteine residues of the Ig-like domains, the extracellular domain of nephrin also contains free cysteine residues that may serve as linkers between binding partner(s) (30).

4.2.2.4 Fibronectin type III-like module
A fibronectin type III-like domain, coded by exons 22 and 23, is found upstream of the putative transmembrane domain, which is coded by exon 24. The fibronectin type III domain was first identified in the extracellular matrix protein fibronectin (74). The domain includes a 90-residue module with conserved tryptophan and tyrosine residues in its amino- and carboxy-terminals. The secondary structure of the domain is highly similar to the Ig-like fold (75, 76). Glycoproteins of the Ig superfamily with fibronectin type III-like domains interact with each other and with other molecules of the extracellular matrix (77-79).

4.2.2.5 Intracellular domain
The putative cytosolic domain is coded by exons 25-29 resulting in a 155-amino acid domain. The presence of eight tyrosine residues in the intracellular domain suggests a role in signalling (see Chapter 4.5). The intracellular domain shows no significant homology to other proteins (30).

4.2.2.6 Nephrin is N-glycosylated
The human nephrin protein sequence has ten putative N-glycosylation sites, i.e. oligosaccharide units can be linked to the asparagine side chains of the protein by N-glycosidic bonds. This has been postulated to occur based on the finding of a difference between the calculated

molecular weight (MW) of nephrin (135 kDa) and the MW identified by PAGE and Western blot analysis (185 kDa) (30). Furthermore, peptide N-glycosidase treatment of the glomerular lysate resulted in a decrease in the MW as verified by Western blotting, and the lack of N-glycosylation affects the proper folding and changes the cellular localization pattern of nephrin (80).

4.4 Interaction partners

Since the wealth of evidence strongly suggested that nephrin is the key backbone molecule of the filtration barrier it has become increasingly important to identify the molecular complexes associated with nephrin and the physiological function of this molecular machinery. Recent studies have revealed CD2-associated protein, podocin and NEPH1 as interacting partners for nephrin

4.4.1 CD2AP

CD2-associated protein (CD2AP) was the first identified protein interacting with the intracellular domain of nephrin (81-83). CD2AP was originally identified from mouse T cells interacting with the cell adhesion protein CD2 (84). Later, identical sequences were found in a gene involved in mouse kidney development (85) and in a gene encoding focal adhesion protein $p130^{Cas}$-associated protein (86). CD2AP is widely expressed in different tissues and is involved in e.g. cytoskeletal polarity of cells and in signalling cascades regulating cell morphology (87). CD2AP-deficient mice develop nephrotic syndrome, similar to the human disease (88). CD2AP binds directly to filamentous actin, and this way links nephrin to the actin cytoskeleton (89).

4.4.2 Podocin

Podocin interacts with the intracellular domain of nephrin. It is an integral membrane protein and belongs to the stomatin superfamily. *NPHS2*, encoding podocin, has been identified as a gene mutated in autosomal recessive steroid-resistant nephrotic syndrome (90-92). Reduced podocin protein levels have been detected in the glomeruli of PA -nephrotic rats (93). So far, podocin has been shown to be expressed exclusively in podocytes (94, 95) and has been localized to the SD (96); it also interacts with CD2AP (92). Podocin has been shown to be anchored in lipid rafts, which are microdomains rich in sphingolipids and cholesterol, in the outer leaflet of the plasma membrane (92, 97). Interaction with podocin affects the signalling properties of nephrin (see Chapter 4.5)

4.4.3 NEPH1

Nephrin has recently been shown to interact with itself and with NEPH1 (98). NEPH1 is a transmembrane protein, and belongs to the nephrin-like Ig superfamily with five extracellular Ig-like domains. In electron microscopic studies, NEPH1 localizes to the lateral margins of podocytes and also to the SD. *NEPH1* knockout mice present with effacement of podocyte foot processes, proteinuria, and the mice die perinatally (99). NEPH1, like nephrin and po-

docin, localizes to lipid rafts. The intracellular sequence of NEPH1 also interacts with podocin (57).

4.5 Proposed functions

Activators of the protein kinase C (PKC) pathway increase nephrin mRNA expression in cell culture indicating that PKC may be the intracellular signalling system regulating nephrin (100). Nephrin is dramatically down-regulated in mice with decreased levels of Wilms' tumor gene 1 (WT-1), suggesting that nephrin acts downstream of WT-1 (101). Nephrin expression triggers the activation of the transcription factor AP-1 (57). Nephrin is localized to lipid rafts, which are membrane micro-domains containing a given set of proteins mediating activation of signalling cascades. Nephrin-containing lipid rafts also contain the antigen for the 27A antibody, a podocyte-specific 9-*O*-acetylated GD3 ganglioside. *In vivo* injection of 27A antibody to rats causes tyrosine phosphorylation of nephrin (97). Recent studies have shown that nephrin's cytoplasmic domain is tyrosine-phosphorylated by the src family kinases (102, 103). Src family kinase Fyn co-precipitates with nephrin and together with Yes fractionates into the lipid raft fraction. Furthermore, Fyn deletion in mice results in morphological alteration of podocyte foot processes (102). Interestingly, clustering of over-expressed nephrin on the cell surface induced its own Src kinase-mediated tyrosine phosphorylation (103). Most resent report demonstrates that both nephrin and CD2AP interact with phosphoinositide 3-OH kinase and together with podocin trigger AKT signalling in podocytes (104). AKT mediates phosphorylation of several target proteins in podocytes, including Bad (105).

4.6 Monoconal antibody 5-1-6 nephropathy model

The monoclonal antibody 5-1-6 (mAb 5-1-6) nephropathy model was introduced by Orisaka *et al*. (106) already in 1988. Recently, it has been concluded that mAb 5-1-6 recognizes an extracellular epitope of the rat nephrin homologue (107). A single intravenous injection of mAb 5-1-6 into rat causes massive proteinuria and reduction in nephrin expression. However, in electron microscopic studies the SD displays normal morphology in mAb 5-1-6-nephropathic glomeruli (108).

4.7 Nephrin-deficient mice models

Nephrin deficient mice show symptoms of proteinuric disease and die perinatally. Morphologically, the majority of the podocytes' foot processes are fused and the remaining interpodocyte junctions lack slit diaphragms (109, 110). Interestingly, already in the heterozygote phenotype, approximately one third of the foot processes are fused with remarkable down-regulation of nephrin-specific mRNA levels (110).

5. Proteins in podocyte's foot processes

The podocyte foot processes have distinct apical and basal compartments along the divisional plane of the slit diaphragm (**Figure 1D**). Each compartment has a special protein composition. The slit diaphragm protein complex represents a size-selective barrier for macromolecules (93, 111). Adhesion proteins in the basal membrane of the foot processes anchor podocytes to the GBM and stabilize them while proteins on the apical side are thought to keep the adjacent cell membranes apart, preserving in this way the cell shape. The cytoskeleton is needed to maintain the overall architecture of the podocytes. This kind of compartmentalization into functional domains has crucial functional properties in glomerular filtration (112). Interestingly, mutations or deletions in proteins that localize to different compartments in the podocyte result in a podocyte phenotype resembling that found in foot process effacement and proteinuria (14, 88, 99, 110, 113, 114). Hamano *et al.* (115) have proposed that alterations in slit proteins can be observed as a sudden plasma protein leak whereas changes in proteins of the basement membrane lead to an insidious plasma protein leak.

Obviously, a majority of the structural and functional protein complexes of podocytes remains to be characterized. The group of proteins known to date to be involved in SD structure and function is summarized below. Some of the protein complexes situated in the apical and basal membrane compartments or in the cytoskeleton, and found crucial for the filtration function, are presented as well.

5.1 Slit diaphragm

The filtration slit membrane, or SD, bridges the gap, referred to as the filtration slit or slit pore, between the adjacent podocyte foot processes (**Figure 1C and D**). The SD forms a zipper-like structure as reported by Rodewald and Karnovsky (116). The architecture of the slit diaphragm is suggested to be mediated by protein-protein interactions across the foot process intercellular junction (117). The SD structure makes a selective sieve that prevents proteins ≥ albumin from leaking into urine. It is estimated that this surface maintains approximately 40% of the hydraulic resistance of the whole filtration barrier (118).

The tight junction protein ZO-1 was the first protein found abundant in the cytosolic domain of the SD area (119). ZO-1 has been demonstrated to interact with the cell-junction components occludin, alpha-catenin and ZO-2 as well as with the cytoskeletal molecules spectrin and F-actin. Recently, interactions between ZO-1 and NEPH family members have been reported with an implication that ZO-1 may serve as an organizer, and recruit the signal transduction components to the SD (120). Alterations in ZO-1 localization and expression have been observed in PA nephrosis and in the 5-1-6 neph-

ropathy of the rat (121). However, in CNF kidneys with an absent nephrin expression, ZO-1 expression remains unaffected (117).

Recently, the transmembrane proteins FAT (122) and P-cadherin (123) have been shown to locate to the SD domain. In *Drosophila*, FAT was found as a tumour suppressor gene shown to participate in cell proliferation. In addition to P-cadherin, also FAT belongs to the cadherin superfamily with a function similar to cell adhesion molecules that mediate homophilic Ca^{2+}-dependent cell-adhesion. All members of the family have characteristic extracellular domains composed of multiple repeats of a cadherin-spesific motif. So far, FAT or P-cadherin have not turned out to be essential for the maintenance of the normal SD. P-cadherin knock-out mice appear with normal glomerular function (124), and normal expression of P-cadherin is detected in the glomeruli of CNF kidneys (117, 124). If the function of other members in the SD protein complex, such as NEPH1, podocin or CD2AP, is disturbed, the structure of the foot processes is altered and detected as an effaced phenotype associated with proteinuria (see above Chapter 4.4).

5.2 Cytoskeleton

An intact submembranous actin cytoskeleton is needed to maintain the podocyte cytoarchitecture (125, 126). In contrast with the dominance of microtubules and intermediate filaments in the cell body and in the primary processes, microfilaments and actin filaments beneath the cell membrane are present in the foot processes (1). Active polymerisation/depolymerisation of actin filaments obviously occurs constantly in podocytes as evidenced e.g. by the presence of the protein complex Arp2/3 and its activator cortactin in the foot processes (127, 128). Arp2/3 binds to actin filaments and allows the growth of new branching filaments (129, 130).

Actin filaments are cross-linked by alpha-actinin, which connects the actin cytoskeleton to the cell membrane. Non-muscle isoforms of alpha-actinin include alpha-actinin-1 and –4. Of these, alpha-actinin-4 is widely expressed (131) and is found also in glomerular podocytes where it localizes to the foot processes (126). Its central role in the foot processes is evident, since mutations in the *ACTN4* gene lead to familial focal segmental glomerulosclerosis (FSGC) (132). The typical morphological findings of this kidney disease include flattening of the podocytes and detachment of foot processes from the GBM (14). In the PA nephrosis, rearrangement of the cytoskeleton is seen (125, 133), and the mRNA expression level of ACTN4 is indeed increased (126), but the corresponding protein level is similar to that in normal rat glomeruli (93). Recently, Kos *et al*. (134) reported on a severe glomerular disease with disrupted podocyte morphology and proteinuria in alpha-actinin-4-deficient mice. Interestingly, despite ubiquitous expression of alpha-

actinin-4 its deficiency resulted in histological abnormalities only in the kidney.

Synaptopodin colocalizes with actin microfilaments in podocyte foot processes (135). Synaptopodin is postulated to be a marker of the mature podocyte (136). Down-regulation of synaptopodin expression in idiopathic nephrotic syndromes and in CNF glomeruli implies its importance for normal podocyte function (137).

5.3 Basal cell membrane region

The basal aspect of the podocytes forms an important cell domain anchoring cells to the GBM. The mechanisms and molecules involved in basal region have only recently been identified.

5.3.1 Dystroglycan protein complex

After synthetization, dystroglycan is post-translationally cleaved into the transmembrane β-dystroglycan and the heavily glycosylated peripheral membrane α-dystroglycan. These two proteins are non-covalently linked. A larger dystroglycan protein complex including dystrophin, syntrophin, β-, δ and ε -sarcoglycan complexes as well as sarcospan, has originally been isolated from skeletal muscle (138, 139). This complex mediates a connection between the extracellular matrix and the actin cytoskeleton (140, 141). Presence and localization of dystroglycan, sarcoglycan, agrin and utrophin (an autosomal homologue of dystrophin) in the kidney could represent an epithelial complex (142-144)

It is postulated that β-dystroglycan binds via utrophin to F-actin in the podocytes (143, 145). However, studies by Durbeej and Campbell (146) show that ε–sarcoglycan and utrophin are not integral components of the kidney's dystroglycan complex in adult, but only in fetal kidney. α-dystroglycan is differently glycosylated in fetal and adult kidney (146) and binds, in addition to the extracellular portion of β-dystroglycan, also to laminin 1 (alpha-5) (146) (142), perlecan and agrin in the GBM (147).

5.3.2 α3β1-integrin complex

Integrins belong to the widely expressed family of cell surface proteins that attach cells to the extracellular matrix. The binding of subunits α and β results in a heterodimer typical of integrins (148). In podocytes, actin, myosin, alpha-actinin, paxillin, vinculin and talin are connected to the GBM at focal contacts (basal cell membrane) through an α3β1-integrin complex (149, 150). The α3β1-integrin complex adheres to the ECM proteins laminin, proteoglycans agrin and perlecan, type I and IV collagen, fibronectin and entactin/nidogen in GBM (151). β1-integrin connects to the actin cytoskeleton via alpha-actinin-1 (152). In mice lacking alpha-3 integrin, early onset of massive proteinuria is observed (113). Remarkable up-regulation in the protein expression level of β1-integrin

has been reported regarding the glomeruli of PA nephrotic rats (93).

5.4 Apical membrane region

5.4.1 Podocalyxin/Ezrin/NHERF2 and WT-1

The apical compartment, the anionic pole of the podocytes, maintains an electrostatic repulsion barrier in the glomeruli. A negative net charge of the adjacent cell surfaces keeps the intercellular space open (153). Repulsion is mainly due to the integral membrane protein, podocalyxin, which is abundantly found in podocytes (154-159). Podocalyxin is highly glycosylated by N- and O-linked carbohydrates, which are heavily sialylated and sulphated; these residues give a negative net charge to the protein (160). Podocalyxin is connected to the actin cytoskeleton through a complex of ezrin and Na+/H+-exchanger regulatory factor 2 (NHERF2) (161). Ezrin is a member of the ezrin-radixin-moesin (ERM) family of cytoskeletal linker proteins involved in the maintenance of epithelial cell polarity (162-164). NHERF2 is a regulator of the plasma membrane protein NHE-3, the epithelial isoform of the Na^+/H^+ exchanger family proteins (165, 166), involved in sodium absorption in renal epithelial cells (167).

The WT-1 transcription factor regulates the expression of podocalyxin in podocytes, (168). In several glomerular diseases, the presence of podocalyxin in the urine correlates with the degree of podocyte injury (169). Podocalyxin gene-deficient mice fail to form foot processes, as well as SD, and die neonatally (114). In the rat PA nephrosis model, the degree of sialylation of podocalyxin is reduced. This, together with decreased podocyte plasmalemmal surface area, leads to reduced total glomerular sialic acid content (170). In addition, podocalyxin-NHERF2-ezrin interaction with the actin cytoskeleton is disrupted in podocytes of the PA nephrosis model (161).

5.4.2 GLEPP1

Glomerular epithelial protein 1 (GLEPP1) is a podocyte-specific receptor membrane protein tyrosine phosphatase (PTPase) proposed to have a role in podocyte function and structure by regulating tyrosine phosphorylation of podocyte proteins (171, 172). Structural changes in the glomeruli of GLEPP1-deficient mice include blunting and stretching of the podocyte foot processes. Furthermore, glomerular nephrin content and glomerular filtration surface area and rate are reduced in these mice. However, there are no detectable changes in the urine albumin levels of GLEPP1 knock-out mice (172). In the rat PA nephrosis model, GLEPP1 protein and mRNA levels are reduced (173).

6. Densin

6.1 Expression profile

Densin was originally purified from a postsynaptic density (PSD) fraction of rat forebrain (174). The PSD is an electron-dense thickening just beneath the postsynaptic membrane.

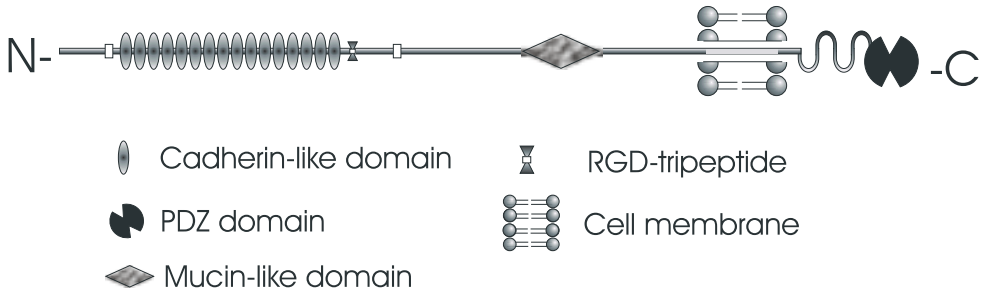


Figure 3. Domain composition of densin. Densin protein sequence predicts an extracellular part, a transmembrane domain and an intracellular part.

This subcellular fraction can be prepared after detergent extraction of synaptosomes (175, 176).The main function of PSD proteins is postulated to be the maintenance of adhesion between presynaptic and postsynaptic membranes at glutamatergic synapses (177, 178).

Densin-180 has shown to be highly brain-specific with higher expression in the forebrain than in the cerebellum (174). The mature protein contains 1495 amino acids with a calculated molecular weight of 167.5 kDa. An apparent transmembrane domain has been suggested (174), but there is still discrepancy as to whether densin is a transmembrane or cytosolic protein (179). Several splicing variants of densin mRNA are expressed (180). One of the splice variants, termed LRRC7, has shown to be ubiquitously expressed (181). The LRRC7 cDNA encodes a putative 216-amino acid protein with a predicted molecular mass of 25 kDa.

6.2. Domain composition

6.2.1 Densin is a member of LAP protein family

Densin is a member of the LAP (leucine-rich repeats (LRRs) and PDZ domains) protein superfamily with 16 leucine-rich repeats, a sialomucin domain and one PDZ domain (**Figure 3**). LAP family members are epithelial proteins with a crucial function in maintaining cell shape and apical-basal polarity. They also play an essential role in carrying their interacting partners to specific plasma membrane domains (182, 183). The LRRs in these proteins are located in the amino terminus with higher relation to each

other than to LRRs of other proteins. In densin, like in other typical LRR-containing adhesion molecules, cysteine-rich domains flank the LRRs. However, the cysteine clusters of densin do not match the typical consensus of LRR-containing proteins (184). In addition to densin, three additional LAP proteins have been reported in vertebrates: erbin (185), scribble (186) and lano (187).

6.2.2 Leucine-rich repeats

Leucine-rich repeats (LRRs) are short, 22- to 28- residue motifs in a variety of cytoplasmic, membranous and extracellular proteins with diverse functions. A common property of the LRR motifs is protein-protein interaction e.g. in hormone-receptor connection, enzyme inhibition, cell adhesion, cellular trafficking and vascular repair (188, 189). According to the three-dimensional structure of the LRR domains, it is possible that they form structures capable of interacting with membranes (190).

6.2.3 PDZ domains

PDZ domains were first identified in the post-synaptic density protein 95 (PSD-95), the *Drosophila melanogaster* discs large protein (DLG) and the zonula occludens 1 protein (ZO-1) contracting to form the name PDZ according to the first letters of the name in these proteins. Additional names for the domain are DHR (according to Discs large Homology Repeat) and GLGF (after the highly conserved four-residue GLGF sequence within the domain). PDZ domains are about 90 residues in length and are conserved structural elements present as single or repeated copies in a variety of proteins (191). The PDZ domain functions as a protein-protein interaction module, and is currently appreciated as a central organizer of protein complexes at the plasma membrane.

PDZ domains are highly sensitive for specific C-terminal peptide motifs. (192, 193). Densin PDZ domain represents type I. The binding of these class I PDZ domains is mediated by a (S/T)X(V/L) (S for serine, T for threonine, V for valine and L for leucine) motifs found in the carboxyl-termini of their binding partners (194). In addition to binding to specific C-terminal sequences, PDZ domains are capable of interacting with internal peptide fragments of the target proteins (195-197). Binding of two PDZ domains on separate proteins could facilitate heterodimerization or complex formation (198). Interleukin-16 is the only secreted member of the PDZ protein family reported thus far (199).

6.2.4 RGD peptide

The Arg-Gly-Asp (RGD) tripeptide sequence was first described in fibronectin as a sequence mediating cell attachment (79, 200) via binding to integrins. Later on, RGD has been identified from several proteins also as a sequence not involved in attachment (201-203).

6.2.5 Mucin-like domain

A specific, 90 amino acids long domain in the extracellular part of densin is rich in serine, threonine and proline (174). The composition of

the sequence is similar to that found in mucines and is termed mucin-like domain. It is present in many proteins and is a target region for O-glycosylation (204).

6.3 Interaction partners

Densin PDZ domain has been shown to interact directly with the actin-binding and crosslinking cytoplasmic protein, alpha-actinin-4 (205). Another reported interaction partner is the (alpha)-subunit of Ca2+/calmodulin-dependent protein kinase II (CaMKII) (174, 180, 205). CaMKII is identified, like densin, as a PSD fraction protein in synapses involved in calcium influx and synaptic plasticity (206, 207). Densin interaction has been shown with adherens junction protein delta-catenin/neural plakophilin-related armadillo repeat protein (NPRAP) at synapses where densin has been observed to co-immunoprecipitate with delta-catenin/NPRAP and N-cadherin. (179). Recently, the densin PDZ domain was reported to interact with the membrane-associated guanylate kinase (MAGUK)-interacting protein MAGUIN-1, a protein that binds to PDZ domains of the synaptic scaffolding molecules (S-SCAM)/MAGI-2 and to another MAGUK protein in the PSD fraction, the 95-kDa postsynaptic density protein (PSD-95) (208).

7. Pancreas

The pancreas is an organ that measures 25 cm long, 5 cm wide and 1-2 cm thick. Histologically it can be divided into exocrine and endocrine parts, both of them involved in secretion of enzymes and hormones (209).

7.1 Exocrine pancreas

The exocrine pancreas consists of special structures called pancreatic acini. Each acinus consists of three to four centroacinal cells surrounded by approximately 40-50 acinar cells. The acinar cells produce large amounts of enzymes including pancreatic amylase, pancreatic lipase, ribonuclease, DNase, proenzymes trypsinogen, chymotrypsinogen, procarboxypeptidase and elastase (210). The cells also produce a trypsin inhibitor to protect the cells from intracellular activation of protein peptidase trypsin. Accidental activation of digestive enzymes within the cytoplasm of the acinar cells leads to serious acute pancreatitis. The centroacinal cells in the acinar lumen contribute to the first part of the duct system by forming intercalated ducts, which outside of the acinus lumen join each other and form intralobural ducts. These ducts then converge to form interlobural ducts that later connect to the main pancreatic duct (209).

7.2 Endocrine pancreas

The endocrine pancreas is mainly composed of special structures called islets of Langerhans. These structures consist of five types of cells that do not exhibit any unusual morphologies but each cell type has different function in the pancreas. The most abundant cell type in the

islets of Langerhans are the insulin-producing β–cells. The β–cells are concentrated to the center but are in minor amounts also found throughout the islets. Secreted insulin hormone binds to its receptor and activates glucose take-up especially on skeletal muscle, liver and adipose cells in order to decrease blood sugar levels (210). Diabetes mellitus is a disease characterized by hyperglycemia, lack of insulin production in the pancreas or inhibition insulin resistance in target cells and tissues. Diabetes often leads to secondary complications, including blindness (diabetic retinopathy), gangrenes (diabetic angiopathy), myocardial infarctions (mmicroangiopathy/endothelial changes) and renal complications (diapetic nephropathy) (210).

One fifth of the islet structure is composed of α-cells, which are located in the periphery. These cells produce the peptide hormone glucagon. In liver hepatocytes glucagon activates the breakdown of glycogen to glucose, thus increasing the blood sugar level (209, 210).

Minor amounts of additional cell types are scattered throughout the islets. δ- cells produce somatostatin hormone, which inhibits the release of endocrine hormones in α-and β–cells. In addition, somatostatin reduces contractions in the alimentary tract and gall bladder smooth muscles. G cells release gastrin that stimulates production of HCl. PP cells produce pancreatic polypeptide that inhibits exocrine secretion of pancreas (209, 210).

Aims of the study

NPHS1 gene, encoding nephrin protein was cloned in 1998 and its mutations were shown to be responsible for the monogenic kidney disease, CNF. Because of the dramatic effect of these mutations, i.e. massive proteinuria, it was obvious that nephrin has an important role in maintaining glomerular filtration function.

The specific aims of the thesis are as follows:

- to clone the rat nephrin homologue
- to show the specific localization of nephrin in glomeruli
- to study the role and functional genetics of nephrin in proteinuric rat models
- to search for nephrin expression in other tissues in addition to the kidney
- to establish nephrin-associated proteins

Materials and methods

1. Tissue samples

All procedures used here were approved by the ethics committee of the Helsinki University Central Hospital, or in the case of animal studies, the ethics committee of the University of Helsinki.

1.2 Normal human and CNF kidneys (II, V)

Cadaver (n = 5; age of donors, 12–48 years) kidney tissues, inappropriate for transplantation because of failure in vascular anatomy, or the intact areas of removed Wilm's tumour kidneys (n = 2; ages, 3 and 5 years) were used (Department of Surgery, University of Helsinki).

To study CNF kidneys, patients with typical symptoms including placental weight >40% of the weight of the newborn, oedema and massive proteinuria, were rendered to kidney nephrectomy as described earlier in a special treatment protocol (39). Other types of congenital nephroses were excluded later based on typical pathology.

After removing the kidney medulla, the cortex was used as such or further prepared for glomerular isolation. Part of the kidney material was always stored at –70°C.

1.3 Human pancreatic tissue and RNA (IV)

Cadaver pancreatic tissues were used (Division of Transplantation Surgery, University of Helsinki, Helsinki, Finland). The isolated islets of Langerhans with 50 – 60 % of beta cells, as well as islet total RNA, were kind gifts from Dr. D. Eizirik (Free University of Brussels, Belgium)

1.4 Human brain tissue (V)

The human brain sample was from normal tissue of a young adult's temporal lobe removed during tumour surgery, provided by Dr. Anders Paetau (University of Helsinki, Faculty of Medicine, Department of Pathology)

2. Rat animal models (I,III)

The rats in the PA nephropathy study were divided into six differentially treated groups (six rats in each group) with a single intraperitoneal injection of 1) PA, 2) $HgCl_2$ or 3) both PA and $HgCl_2$. One of the groups was treated with 4) daily doses of the antioxidant agent probucol 10 days before the PA + $HgCl_2$ injection and one with 5) daily doses of probucol following injection, not with PA and $HgCl_2$ but with 0,9 % saline in equal amounts. The control animals 6) were injected with saline alone. Following sample collection and analysis are detailed in the original work III.

Autoimmune Heymann nephritis and nitric oxide synthase variation were induced in the rats

as described by Uhlenius *et al*. (211) and in the original publication (Study III).

In all settings mentioned above, after the removal of the kidney medulla, the cortex was used as such for mRNA isolation. Part of the cortex material was stored at –70°C for immunofluorescence and immunoelectron microscopy studies.

3. Glomerular isolation

Glomeruli from human and rat cortex were isolated using the sieving method (212, 213). Fresh or frozen cortex was cut into macroscopic homogeneity in ice-cold PBS. The resulting tissue homogenate was pressed through the 250-µm and 150-µm sieves by rinses with ice-cold PBS. Glomeruli were collected from the 106-µm and 75-µm filters, and the purity of the isolation was checked under light microscope. A typical purity for glomeruli collected from the 106-µm sieve was about 90 %, whereas much higher purity was found for the glomeruli collected from the 75-µm sieve.

4. Cell culture

Human podocytes were isolated from normal human kidney as described earlier (214, 215) Briefly, isolated glomeruli were suspended in D-MEM containing 10% heat-inactivated FCS, 2.5 mM glutamine, 0.1 mM sodium pyruvate, 5 mM HEPES buffer, 1 mg/ml streptomycin, 100 U/ml penicillin, nonessential amino acids (0,1x dilution made from 100x; all Seromed, Berlin, Germany), insulin, transferrin, and a 5-mM sodium selenite supplement, and were incubated at 37°C (5% of CO_2). Cell colonies sprouted around the glomeruli were excised and incubated in 5 ml of 0.2% collagenase IV (Sigma-Aldrich, Deisenhofen, Germany) at 37°C for 30 min followed by washes and further plating.
The cells showed an epithelial morphology and stained positive for the Wilm's tumour Ag (WT1) and nephrin, markers found only in podocytes of the adult kidney. The cells were also negative for the endothelial cell marker factor VIII-related antigens.

5. Antibodies

Production and characterization of anti-nephrin primary antibodies is described in detail in the original publications I-V and anti-densin primary antibodies in reference (179). Commercial primary and secondary antibodies are described in the original publications as well.

6. Molecular biological methods

RNA- or DNA-based molecular biological methods used in the original publications of this thesis are summarized in table I.

Table I. Molecular biological methods used in this thesis

Methods	Original publi-cations	References
Analysis for the Fin_{major} (in exon 2) and Fin_{minor} (exon 26) mutations in *NPHS1* gene	II	(30)
RNA isolation and cDNA synthesis	I, II, III, IV, V	
Reverse transcription-polymerase chain reaction (RT-PCR) with		
1) Rat nephrin primer sequences	I, III	
2) Human nephrin primer sequences	II, IV	
3) Human densin primer sequences	V	
4) Rat densin primer sequences	V	
Semiquantitative (RT-PCR)	I, III, V	(216)
Sequencing of PCR products or isolated plasmids	I, II, III, IV, V	
Human tissue dot blot	IV	
Construction of the probe for the 3' end of human nephrin (HN3') and the 5' end of rat nephrin (RN5')	I, IV	

7. Cloning of rat nephrin cDNA (I)

Cloning of the rat nephrin homologue was performed in order to produce tools for studying nephrin in rat models. For that purpose, a rat glomerular cDNA library (217) was used to achieve a greater portion of mRNA originated from podocytes. The library was plated onto petri-dish and further transferred onto a nylon membrane (Hybond N+; Amersham Life Sciences, Buckinghamshire, England), according to the instructions of the manufacturer. Prehybridization was performed at +42° C followed by hybridization in the hybridization solution containing the HN3' probe labelled with [alpha-^{32}P] CTP (3000 Ci/mmol, 10 mCi/ml; Amersham). After an overnight hybridization, the filter was washed and exposed onto the film (Fuji Photo Film, Japan). Positive plaques were picked up and used for successive rounds of screening with the nephrin-specific probe HN5'. After the single-clone excision protocol (ZAP Express cDNA Gigapack II Cloning kit;

Stratagene), the DNA of the pBK-CMV phagemid clone was isolated (Quiagen plasmid Mini Kit; Quiagen, Hilden, Germany) and sequenced from three clones along both strands, and the sequences were screened for homology with database sequences, using the BLAST search algorithm at the National Center for Biotechnology (Washington DC, USA)

8. Immunochemical techniques

8.1 Immunofluorescence (I,II,III,IV,V)

Frozen tissue sections were cut at 5 µm and fixed with acetone, methanol or paraformaldehyde (PFA). After incubation with the respective primary antibody a FITC-or –TRITC-conjugated secondary antibody was used. Washes after fixation and antibody incubations were performed five times in PBS. Finally, Immu-Mount embedding medium-covered (Shandon, Pittsburgh, PA) sections were analysed with an Olympus OX50 fluorescence microscope (Olympus Optical GmbH, Hamburg, Germany).

8.2 Immunoelectron Microscopy (II, III)

Immunoelectron microscopy was performed as described earlier (218). Briefly, fixation of normal cortex was employed for six hours in 4% formaldehyde in phosphate-buffered saline at 20°C. After embedding with Lowicryl K4M (Chemische Werke LOW1,Waldkraiburg, Germany), 1-micron sections were cut. These sections were blocked with 1 % ovalbumin for 1 hour followed by incubation with the primary antibodies and the relevant 10-nm gold conjugate (1:50).

8.3 Immunoblotting (I,II,III,IV,V)

Protein lysates were suspended into reducing Laemmli buffer (62,5mM Tris-HCl (pH 6,8), 10% glycerol, 2% SDS, 5 % 2-mercaptoethanol, 0,05 % bromophenol blue) and heat-denaturated at 100 °C for 10 minutes. The proteins were electrophoresed in sodium dodecyl sulphate (SDS) polyacrylamide gel under reducing conditions and electrotransferred onto a nitrocellulose membrane (Scleicher & Schuell, Dassel, Germany). Unspecific binding was blocked by incubation in 3% bovine serum albumin or 5 % fat free milk. Primary antibody diluted in the same solution was hybridized, this followed by incubation with the horseradish peroxidase-conjugated secondary antibody. Washings between and after the antibody incubations were performed in PBS. Visualizations of the bound antibodies were performed using the ECL blotting kit (Amersham LifeScience, Amersham International, Buckinghamshire, GB) or the SuperSignal®West Pico Chemiluminescence Substrate kit (Pierce, Rockford, USA).

8.4 Immunoprecipitations (II, V)

Possible interactions of occluding and ZO-1 with nephrin were studied by co-immunoprecipitations in study II. For immunoprecipitation, the glomerular lysate (1 mg/ml) in radioimmunoprecipitation assay buffer was in-

cubated with antibodies against occludin or ZO-1, using 10µg of IgG/200 µl of glomerular lysate at 4°C overnight. Immune complexes were collected with protein-A-Sepharose (Pharmacia LKB Biotechnology), washed, and processed for immunoblotting with anti-nephrin antibodies.

In order to find interacting partners for nephrin, co-immunoprecipitation with anti-nephrin antibodies was performed. Thus, freshly-isolated rat or frozen human glomeruli were homogenized in TNE buffer (250 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.4) with 0,2% Triton X-100 under +4°C, and the resulting homogenate was pelleted by centrifugation and homogenized similarly once more. The supernatant and the pellet homogenate were pooled and passed through a 25G needle. After a 45-minute incubation on ice the lysate was centrifuged at 14000 rpm with an Eppendorf centrifuge followed by supernatant preclearing by Sepharose 4B protA beeds for 1 hour at +4°C. Protein A beeds were collected and 20µl of anti-nephrin antiserum were added, #050 (see Study V) to the rat trial and #1109 (see Study V) to the human glomerular sample. After an o/n incubation at +4°C, protein A was added followed by washes with homogenization buffer. Finally, Laemmli sample buffer was added to the washed beeds and the mixture was heated for 10 min at 100°C followed by SDS-PAGE.

9. MALDI-TOF-MS (V)

9.1 Sample preparation

Samples immunoprecipitated with anti-nephrin antibodies from rat glomeruli were applied on 8% SDS-PAGE and stained according to Blum *et al.* (219). The stained protein band close to 200 kDa of size was excised from the gel and further cut into small pieces followed by the treatment earlier described by O'Connel and Stults (220). Briefly, the stain was removed with 0,2 M NH_4HCO_3/ACN solution (1:1) at 37°C for 45 min followed by an additional ACN treatment and drying in a vacuum centrifuge. The disulphide bonds were reduced using 55 mM iodoacetamide in 0,1 M NH_4HCO_3 at RT in the dark. The pieces were washed using 0,1 M NH_4HCO_3 and H_2O with ACN shrinking between and final drying under vacuum. Trypsin digestion was performed o/n at 37°C with 0,05 µg/µl sequencing grade-modified trypsin (Promega, Madison, USA) followed by peptide extraction from the gel by 0,1% TFA/ 60% ACN 30 min at 37°C. After additional 0,1 % TFA treatment, the peptides were desalted in a Zip tip and β-cyano4-hydroxy cinnamic acid was added. This mixture was used in the mass analysis.

9.2 Peptide mass analysis

Fingerprinting for the trypsinized peptides was performed with Biflex™ mass spectrometer (2GHZ digitizer) (Bruker, Rheinstetten, Germany) at the Protein Chemistry unit in Bio-

medicum, University of Helsinki. A positive ion reflector mode was used with an accelerating voltage of 19 000 V, and a delayed extraction of 2 ns. Internal peptide calibration standards (Bruker Daltons, Bremen, Germany) were applied to obtain higher peptide mass accuracy. The list of peptide masses obtained in MALDI-TOF-MS analysis was used to search the NCBI database using the ProFound software (Version 4.10.5, Rockefeller University).

Results

1. Rat nephrin cDNA

A cDNA library prepared from one-month old isolated rat glomeruli was used to clone the cDNA transcript with homology to human nephrin. With a human 5'-probe several positive clones were found and by using a rat-specific 5'-probe, a full-length clone was obtained. The 1234-bp coding region of the full-length transcript revealed 83 % identity with the open reading frame of the human nephrin sequence (30) while the predicted aa sequence was 89% identical with human nephrin (**Study 1, Figure1**). The transmembrane domain showed the highest identity, 99%, whereas the intracellular domain showed 83 % identity, and the score for the extracellular domain was 90 %. The predicted structural Ig-like domains were highly conserved, as well as the fibronectin type III-like module. Up to 10 putative N-glycosylation sites were found and were identically localized between human and rat. Also a putative signal sequence was found in the N-terminus of both nephrins. The locations of the cysteine residues were also highly identical between human and rat with the exception that the rat sequence had two additional cysteines at positions 192 and 356.

In the immunofluorescence studies utilizing the intracellular nephrin peptide antibody, a typical epithelial staining was demonstrated in rat glomeruli (**Study I, Figure 5 and, Study III, Figure 4A**). Also in Western blot analysis, a band with an equal size to human nephrin (30) was observed from isolated rat glomerular lysate (**Study I, Figure 6 and Study V, Figure 1A**). With RT-PCR studies of rat kidney, a strong expression of nephrin-specific mRNA was detected (**Study I, Figure 3**). These results demostrated that the found clone had homology to human nephrin and that nephrin is also expressed in rat glomeruli.

2. Nephrin splicing variants

In addition to the whole length sequence, alternatively spliced transcript forms of nephrin mRNA, nephrin -α, –β, –γ and -δ could be detected in the rat (**Study I, Figure 2**). According to sequence analysis, nephrin -α and –β could putatively be translated in a correct open reading frame. When compared to the human sequence (35), α-nephrin lacks the transmembrane domain coding for exon 24 and for the intracellular exons 25, 26 and 27. β-nephrin lacks part of the exon 24 (bp 3166 – 3205). α–nephrin was also found to be expressed in human, but in contrast to rat, it lacks specifically and only the transmembrane-coding exon 24 (**Study II, Figure 5**).

3. Nephrin localization

Immunohistochemical studies of human or rat cortex sections with an antibody directed against the intracellular portion of nephrin showed glomerular staining while the basement membrane, the endothelium, mesangium and tubuli remained unstained. Positive staining of glomeruli was detected in the elements facing the urinary space visualized by finely granular dotted lines preferentially seen in staining with podocyte-specific antibodies (**Study I, Figure 5B and Study II, Figure 1B**). Further electron microscopic studies with the same anti-nephrin antibodies revealed labelling of podocyte foot processes at the level of the SD (**Study II, Figure 2 and Study III, Figure 5**). Some minor accumulation was detected also in the plasma membrane of the apical surface of the secondary processes.

4. Nephrin involvement in animal models of proteinuria

Animals treated with puromycin aminonucleoside (PA) started to develop albuminuria at day 3, reaching a maximum at day 9 after the PA injection (**Study III, Figure 1**). In nephrin mRNA expression, a 40% down-regulation at day 3 ($P = 0.037$) and an 80% down-regulation at day 10 ($P = 0.027$) were detected (**Study I, Figure 4; Study III, Figure 2 and Study III, Figure 3**). A decrease in nephrin mRNA level was also seen in the mercuric chloride-treated rats (**Study III, Figure 2 and Study III, Figure 3**), even though with the dosage of $HgCl_2$ used, the urinary albumin did not exceed 1.5 mg/day (**Study III, Figure 1**). This rate is close to that of the controls in the group with combined PA and mercuric chloride treatments. In this combination group an even more distinct down-regulation in the nephrin mRNA level was detected as early as at day 3, while the level of urinary protein remained unaltered. Pretreatment with probucol did not prevent proteinuria in the PA/$HgCl_2$ group. With the probucol dosage used alone the resulting changes in the nephrin mRNA level remained negligible (1.4- and 1.35-fold increase) at days 3 and 10, respectively. In Heymann nephritis, untreated rats or those treated with a NOS inhibitor failed to show any significant changes in nephrin mRNA expression levels.

In immunohistochemistry (**Study III, Figure 4 and Table 2**), an irregular and less intense pattern of reactivity with anti-intracellular nephrin antibodies was observed in the PA nephrosis group in the sample taken at day 10, whereas insignificant changes were detected at day 3. Only a slight change in staining pattern was observed in the Heymann nephritis and $HgCl_2$ groups. End-point titration was used in semi-quantitation and two independent observers measured the staining intensity in tissues.

As mentioned above, in immunoelectron microscopy the anti-intracellular nephrin antibod-

ies primarily recognized the filtration slit domain in podocytes. However, 27 % of the immunogold particles were regularly observed also along the apical plasma membrane. In PA nephritic rats, a significantly increased amount (87%) of the particles was detected on the apical surface (**Study III, Table 3**). In Heymann nephritic kidney, the gold particles were also found in intracellular vacuoles (**Study III, Figure 6**).

5. Expression profile of nephrin

The tissue distribution studies of nephrin with the human RNA Masterblot and with RT-PCR revealed mRNA expression, in addition to human foetal and adult kidney, in the human pancreas and in the rat spleen (**Study I, Figure 3 and Study IV, Figure 1**). In the pancreas, nephrin and α-nephrin mRNA expressions were detected in the islets of Langerhans (**Study IV, Figure 2**). The dominant form in the pancreas as well as in the kidney was the full-length nephrin.

In immunoblotting studies with anti-nephrin antibodies, a band at 165 kDa was detected from the pancreas and the islets of Langerhans (**Study IV, Figure 3**). In immunofluorescence studies, nephrin localized to the islets of Langerhans, specifically to insulin-positive beta-cells (**Study IV, Figure 4**). The immunoreactivity in Western blot and immunofluorescence analysis was abolished by pre-incubation of the anti-nephrin antibody with the corresponding antigen.

6. Nephrin-associated molecules

In order to search for additional podocyte molecules interacting with nephrin immunoprecipitation of rat glomerular lysates with anti-nephrin antibodies was done. Proteins of the precipitate were separated in SDS-PAGE, silver stained and identified with MALDI-TOF mass spectrometry. Of the peptide masses obtained, 11 matched with rat densin originally identified from the forebrain and thought to be expressed exclusively in the brain.

RT-PCR analysis showed densin mRNA expression in glomeruli as well as in cultured human podocytes (**Study V, Figure 2**). cDNA prepared from rat brain tissue was used as a control. Sequencing of the amplified PCR products of brain and podocytes confirmed 100 % identity with densin. In Western blot analysis, the anti-densin antibody reacted with the antigen of 210 kDa in human glomerular lysate, while a 185-kDa band was obtained from the lysate of cultured human podocytes (**Study V, Figure 3A**). Immunoblotting of human brain lysate revealed the corresponding 185-kDa band as expected based on previous studies by Izawa *et al.* (179). In addition to the interaction found for the rat, co-immunoprecipitation studies with the anti-nephrin antibody of human glomerular lysate confirmed this association of nephrin

with densin in human, as well (**Study V, Figure 3 B**).

Immunofluorescence studies of semithin frozen sections using anti-densin antibody revealed intensive glomerular staining of podocytes (**Study V, Figure 5A and B**) and faint staining was also observed in proximal tubuli (**Study V, Figure 5C**). In immunoelectron microscopy (**Study VI, Figure 6A, B and C**), immunogold particles of anti-densin antibody were located to the slit diaphragm domains.

Increased densin mRNA expression in CNF kidney cortex was detected by semiquantitative RT-PCR when compared to normal human cortex (**Study V, Figure 4A**). Similarly, increased densin protein expression was revealed in CNF glomeruli by semiquantitative immunoblotting (**Study V, Figure 4 B**). However, in immunofluorescence studies, anti-densin antibodies showed reduced staining in CNF glomeruli, while their staining intensity with anti-podocalyxin antibodies remained unchanged between CNF and normal tissues (**Study V, Table I**).

Discussion

1. Rat homologue of nephrin

In order to establish the role of nephrin in well-characterized rat animal models of proteinuria we cloned the rat nephrin sequence. High similarity at the amino acid level as well as highly conserved domains suggested that the found rat cDNA was a homologue of the respective human nephrin cDNA. Later, other groups have cloned similar sequences of the rat (221, 222). All three separate amino acid sequences are almost identical apart from the signal sequence with the exception that sequence found in this study (accession number AF125521) includes asparagine at position 1218, whereas in the other 2 sequences it is aspartic acid. From the signal sequence we reported originally the first methionine 18 amino acids downstream from that in the other 2 sequences. However, in later studies (unpublished) we found the identical methionine and the first 17 amino acids also from our clone (**Figure 4, AF125521**). Interestingly, Kawachi *et al.* (221) reported threonine at the second methionine position (**Figure 4, AF161715**). This could be explained as a sequencing artifact, but since other species (human and mouse) have the same amino acid in that position, it is most unlikely. Thus, there might be differentially expressed forms of nephrin that use different methionine codons for translation.

HUMAN (AF035835) **M**ALGTTLRASLLLLGLLTEGLA

RAT(AF125521) MGAKRVTVRGARTSPIHR**M**SSLTPLLLMGMLTSGLA
RAT(AF172255): ----------
RAT(AF161715) ----------T----------

MOUSE(AF172256):**M**GAKEVTVRGPGASPVHRTCRLIPLLLAGMLTTGLA
MOUSE(AF168466): **M**ALGTTLRAS----------

Figure 4. Comparison of N-terminal signal sequences of nephrin according to different reports and species. The first methionine is bolded and the previously unpublished sequence is underlined. Sequences are numbered with the corresponding Genebank accession number.

These changes are interesting also because of significant variation in two separately reported mouse sequences (222, 223) in that region. Holzman *et al*. (223) reported on the first methionine of mouse in the same position as in the human sequence (**Figure 4, AF168466**) while Putaala *et al*. (222) reported on identical lengths of mouse and rat N-terminal signal sequences (**Figure 4, AF172256**). Holzman *et al.* (223) also suggested the first 10 amino acids of their clone to be identical to the human sequence, but Putaala *et al.* (222) reported on completely different amino acids in the corresponding region.

2. Nephrin-specific mRNA is differentially spliced

In order to be translated into proteins, genes are first translated into non-functional pre-mRNA followed by splicing that removes introns and splices exons together producing final mRNA. However, as for alternative splicing, different exons can be included or excluded and thus several different mRNAs may be produced from a single gene with the ability to be translated into proteins. The picture is physiologically rather complicated since it has been estimated that almost 60% of genes may have splice variants and, many of them, more than one (224, 225).

In addition to the full-length nephrin we were able to isolate differentially transcribed clones from the rat glomeruli. The most interesting one and also the most abundant one after the whole-length transcript is α–nephrin that lacks the transmembrane domain coded by exon 24, as well as for exons 25, 26 and 27 (numbering according to the human *NPHS1* (35). A transcript without exon 24 was obtained also from human indicating possible presence of soluble nephrin protein. Involvement of soluble nephrin in glomerular filtration is assumed since in the PA neprotic rats, nephrin-α mRNA showed similar down-regulation to the full-length transcript (226). In the same study, nephrin protein was detected from the proteinuric urine samples of the corresponding rat model. However, it remains to be shown whether the detected nephrin in the urine represents the soluble form or whether it is released to the urine by some other mechanism like limited proteolysis. The importance of the soluble forms of transmembrane proteins has been found for example for the transmembrane-negative splice variant of vascular endothelial cell growth factor receptor inhibiting the respective ligand activity (227). Similar functionally important isoforms lacking the transmembrane domain have been found, for instance for T-cell receptor (228) and the interleukin 6 receptor (229) as well as for the endothelial angiotensin converting enzyme (230).

Recent studies have revealed an additional exon 1A in the *NPHS1* gene and different tissue expression patterns of nephrin-specific mRNA under different lengths of the *NPHS1* promoter segment (56). This may implicate more com-

plex splicing combinations and thus involvement of different isoforms of nephrin protein in different tissues and physiological conditions.

3. Altered nephrin expression in experimental animal models

The ethiology and pathogenesis of proteinuria are poorly understood. The molecular mechanisms of the filtration function have been studied for years e.g. by using a variety of experimental animal models. These rat models are well characterized and are in that sense still highly usable. Moreover, since the crucial importance of glomerular filtration function for the whole body homeostasis, several filtration barrier gene-specific deficiencies of mice are lethal, and thus rat models are needed to provide samples from early and late stages of the disease.

In studies regarding nephrin expression level, remarkable down-regulation of corresponding mRNAs and proteins was detected in PA nephrosis. A similar change has been reported e.g. in the acquired human nephrotic syndrome (231). This and particularly the finding that nephrin mRNA is reduced already before the onset of proteinuria indicate early nephrin involvement in the disease process. Its close association in the molecular mechanisms of disease is supported also by the obvious redistribution of nephrin between the apical plasma membrane and the SD in PA neprotic rats.

Involvement of free oxygen radicals in nephrin regulation was studied by treating PA nephrotic animals with the antioxidant probucol. This treatment, however, failed to rescue normal nephrin mRNA expression. On the other hand, the dosage of probucol used alone appeared to slightly increase the level of nephrin mRNA. This may suggest certain degree of lipid peroxidation in the appearance of nephrin expression in podocytes e.g. via effects on the intracellular signalling cascades.

In Heymann nephritis, nephrin expression level remained unchanged. This might occur due to late sacrifice (12 weeks after the induction of disease) of the treated animals. At that time point, the possible damages may already have been missed as the long-term balance in the molecular complexes has obviously been reached. Thus, samples in this model should most probably be obtained at earlier time points to reflect the initiating primary events in the perturbation of the functional filtration barrier.

4. Abnormal nephrin and densin expression in CNF kidneys

Using PCR primers for flanking the transmembrane domain of nephrin, two reaction products could be observed both in normal and CNF glomeruli by RT-PCR. In immunofluorescent studies using anti-intra or anti-extracellular nephrin peptide antibodies, all but one CNF kidney failed to show reactivity (**Study II**).

Moreover, nephrin immunoreactivity was detected in EM analyses of some of the CNF samples studied, preferentially at the plasma membranes. This result seems controversial since, particularly in Fin_{major}, no protein should be expressed (35). On the other hand, only homozygosity in Fin_{major} mutations (early stop codon in exon 2) of *NPHS1* should lead to complete loss of nephrin. The Fin_{minor} mutation found in 20% of Finnish patients causes a nonsense mutation at exon 26 beyond the recognition site of intracellular antibodies used in IEM, and antibodies should normally also detect this product. Thus, the Fin_{major}/ Fin_{minor} may produce nephrin detectable only in IEM analysis but not in indirect immunofluorescence microscopy. Also, in our semiquantitative RT-PCR studies (unpublished), a 50-percent reduction in nephrin mRNA level was detected in CNF glomeruli, which may contribute to the reduced protein level as well. A CNF patient with glomerular reactivity in IF studies failed to show neither the Fin_{major} nor Fin_{minor} mutation in direct sequencing. Hence, this patient may present with other mutations in *NPHS1* or in the respective promoter area.

Most probably, critical mutations in some nephrin-associated proteins might also lead to disturbed function of nephrin protein even if nephrin itself remained normally expressed. The importance of the interactions found for nephrin is supported by the fact that in CNF glomeruli, densin mRNA and protein expression were increased, evidenced by semiquantitative RT-PCR and Western blotting when compared to normal glomeruli. However, the glomerular immunostaining for densin was reduced. This apparent controversy may reflect an abnormal distribution and localization of densin in the flattened podocytes due to the absence of SD and nephrin in CNF glomeruli preventing proper anchoring.

5. Pancreatic nephrin expression

In addition to kidney glomeruli, nephrin expression was detected in the islets of Langerhans, particularly in the insulin-producing β-cells. In Western blot analysis, the affinity-purified nephrin antibody revealed two distinct bands from glomerulus with approximate MWs of 185 and 165 kDa. However, in the pancreas, only the 165-kDa band was detected. This is most probably due to different glycosylation patterns of nephrin (30, 80). However, differently spliced forms of nephrin mRNA were observed in this thesis implicating the presence of different protein forms of nephrin as well. Furthermore, Beltcheva *et al.*(56) reported on a novel exon upstream from the originally found exon 1 in *NPHS1*. This exon is transcribed in a specific, tissue-dependent manner. Also, different promoter fragments have been shown to facilitate nephrin expression in different tissues (95). Thus, the lower migration band of pancreatic

nephrin might depend on varying mRNA splicing.

Mutations in the *NPHS1* gene cause dramatic changes in glomerular function as demonstrated in CNF (30). Beta-cell function in CNF pancreas has not been studied, and CNF patients do not show any symptoms of disturbed function of the pancreas. However, after kidney transplantation, the patients undergo heavy immunosuppressive therapy, which has multiple metabolic effects and thus can prevent outcome in the pancreas.

Streptozotocin (STZ)-induced insulin-dependent diabetes mellitus in the rat is an animal model of diabetes associated with proteinuria (232, 233), and increased expression of the glomerular nephrin-specific mRNA (234). In light microscopy studies using anti-nephrin antibodies, a less intense reactivity at the epithelial region has been detected in STZ rats when compared to normal rat glomeruli. Furthermore, nephrin protein is lost to the urine in these rats (234). Whether the disease development in this model involves pancreatic nephrin remains to be investigated.

Later *in situ* studies have shown high nephrin mRNA expression also in the hindbrain and in the developing spinal cord of mouse (222). LacZ-gene transgenic mice showed lacZ expression in the kidney, brain and pancreas under the *NPHS1* gene promoter activity. Nephrin protein expression has also been verified in the mouse brain by immunohistochemical staining (109). In the studies by Liu *et al.* (235), nephrin mRNA and protein expression were detected in the mouse testis, particularly in the Sertoli cells. Moreover, our recent studies have revealed nephrin expression in selected cell types of the human lymphoid tissues (unpublished).

In studies with transgenic mice it has been shown that different fragments of the nephrin promoter region are responsible for nephrin expression in different tissues. Interestingly, the omission of exon 1B of the mouse *NPHS1* gene resulted in expression only in the kidney and brain whereas deletion of exon 1A launches a brain- and spinal cord-specific nephrin expression (56).

6. Architecture of the slit diaphragm (SD)

Electron microscopic results show that nephrin is clearly located at the filtration slit area and is in smaller amounts also present on the apical plasma membrane of podocytes. A similar localization pattern has been shown by other investigators as well (222, 223, 236). Also densin, a novel interaction partner of nephrin, obtained in co-immunoprecipitation studies with anti-nephrin antibodies, localized particularly to the slit diaphragm domain. Thus, two additional members of the SD protein complex were found in this thesis (**Figure 5**).

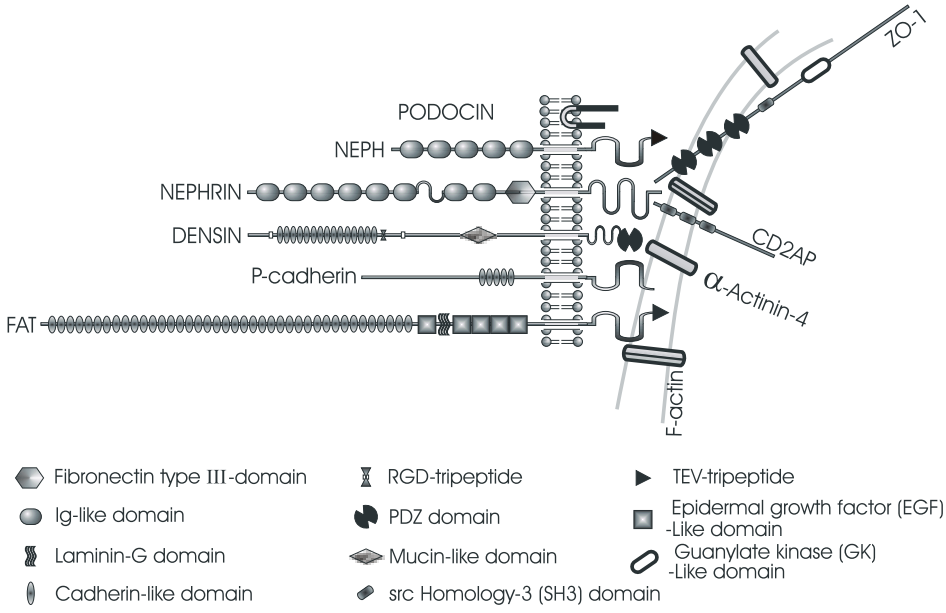


Figure 5. Schematic illustration of the molecular machinery in the slit diaphragm (SD). NEPH indicates nephrin-like molecules NEPH-1, NEPH-2 and NEPH-3/filtrin.

We first proposed that the interaction of nephrin and densin may occur due to a larger protein complex. The proposal is supported by the fact that in the lysate, most of the membrane proteins are still insoluble and larger membrane complexes remain in the supernatant allowing precipitation of proteins without direct interactions. However, recent studies in our laboratory have revealed direct interaction between the intracellular regions of nephrin and densin proteins (unpublished). This is interesting particularly because of postulated functions of LAP proteins in the maintenance of apical-basal polarity in the cell (182). Thus, densin may direct other proteins, like nephrin, to localize at the SD. A similar function has also been proposed

for another PDZ domain-containing protein in the SD, ZO-1 (**Figure 5**).

The splice variant LRRC7 of densin shows ubiquitous mRNA expression profile in human organs, including the kidney (181). However, the putative size of the protein encoded by LRRC7 cDNA is only 25 kDa and the anti-densin antibody and densin sequence specific primers used in this thesis are directed outside of LRRC7 coding region. Thus, the possible role of this splice variant in the kidney remains to be studied.

The transmembranous nature of nephrin with an extracellular and a characteristic intracellular domain has shown to reflect functionality. The extracellular domain most likely mediates outside-in signalling (97). The intracellular part of nephrin mediates its binding to the cytoskeleton via CD2AP (81, 82) and directly to the membrane proteins podocin and NEPH1 (57, 98). Since alpha-actinin-4 has been directly shown to interact with the densin PDZ domain (205), interaction of densin and nephrin may offer a novel link between the SD protein complex and the actin cytoskeleton. The intermediary role of alpha-actinin-4 appears important for the cytoarchitecture of podocytes (126) and mutations in the *ACTN4* gene lead to familial focal segmental glomerulosclerosis (FSGC) (132). Furthermore, *ACTN4*-deficient mice develop severe abnormalities only in podocytes, demonstrating its importance in maintaining podocyte stability (134).

The preferential association of nephrin to the SD and particularly to the detergent-resistant membrane domains, i.e. lipid rafts, and nephrin interactions with lipid raft proteins podocin and NEPH1 suggest that lipid rafts are crucial in the SD architecture (92, 97). However, in our studies densin failed to associate in the lipid rafts, but did instead in the high sucrose fractions after sucrose gradient centrifugation of glomerular lysate (data not shown). Interestingly, the main pool of nephrin was also localized in these high sucrose fractions (97). So far, SD proteins P-cadherin and FAT have not been reported to localize into lipid rafts.

Hypothetical models of SD structure based on homophilic interactions between SD proteins of the adjacent secondary processes have been presented. Nephrin structure with its eight Ig-like domains capable of homophilic interactions has been proposed to reflect its role as a backbone of the SD structure by connecting the adjacent foot processes (237). The proposed function of densin in bridging presynaptic membranes to postsynaptic ones in the glutamatergic synapses also remains intriguing: the physical dimensions match closely to the interpodocyte distance separated by the slit diaphragm. Homotypic adhesion found in some LRR-containing proteins also resembles the proposed functions

of nephrin as an interpodocyte spacer. Similarly, cadherin-like domains in the extracellular domain of FAT and P-cadherin (**Figure 5**) may theoretically mediate a homophilic connection across the slit pore. Moreover, it is most likely that the extracellular domains of SD proteins mediate also heterodimeric interactions as has already been shown for nephrin and NEPH1 (98). RGD peptides in NEPH family members and in densin could mediate binding with integrins. Also, cysteine residues in densin and nephrin predict the presence of additional extracellular interactions.

The intracellular region of CD2AP consists of SH3-like domains that are shown to bind typically to proline-rich regions of interacting proteins. Interestingly, the intracellular domains of the NEPH family members, FAT and P-cadherin, are abundant in proline and therefore this may indicate more interactions in the cytosolic part of the SD. Also, a typical function for the type I PDZ domains presented in densin and ZO-1 is to bind to distinct C-terminal sequences of other proteins (194). It is of particular interest that the SD components FAT and the NEPH family members contain the three typical C-terminal amino acids (TEV) interacting with the PDZ I-type domains (194). Thus far, interactions between the PDZ domain in ZO-1 and the TEV sequence in NEPH family members have been observed (120). Whether densin or ZO-1 interact with FAT, or densin with NEPH family members, remain to be studied.

Summary and future perspectives

The kidney glomerulus maintains the crucially important filtration function (urine formation) by forming a barrier between the blood circulation and the tubular system responsible for formation of the final urine. The filtration barrier consists of three separate layers: the fenestrated endothelium covering the inner aspect of capillaries, the glomerular basement membrane and, finally, the specialized epithelial cells, podocytes. The ability of podocytes to form long extensions called foot processes with unique intercellular structures called slit diaphragm (SD) reflects a special role in glomerular filtration.

NPHS1 gene was detected in 1998 by positional cloning of the gene responsible for congenital nephrotic syndrome of the Finnish type (CNF). Earlier morphological studies had shown an absence of the final sieve of glomerular filtration barrier, the slit diaphragms (SD), in CNF glomeruli. Thus, involvement of nephrin in podocyte derived filtration function was suspected. In this thesis nephrin was shown to localize to the SD region in podocytes. The rat homologue of nephrin was cloned and its involvement in an experimental rat model, PA nephrosis, was observed. In addition to glomerular podocytes, nephrin expression was detected in pancreatic β-cells and in the spleen. In the interaction studies of nephrin, the synopse-specific protein densin was detected for the first time outside of the brain, in the podocytes. In addition to its interaction with nephrin, densin was also localized to the SD domain in the podocytes.

The composition of the protein complex forming the SD has been basically unknown until the discovery of the *NPHS1* gene. Nephrin localization to the SD followed by detection of nephrin interacting proteins has brought new understanding of the pathophysiological mechanisms of glomerular filtration, especially the role of podocytes and SD in this barrier. It is expected that the information will lead to a deeper understanding of the disease mechanisms in the kidney glomeruli. New understanding is urgently needed since kidney diseases are not only difficult for the patients but are also enormously costly to treat.

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